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[Continued on next page]

(54) Title: PECAM-1 MODULATION

A (i) Blot: anti-Tyr(P)
Blot: anti-PECAM-1

(ii) PECAM-1 XL
90s

B (i) Collagen 100µg/ml IgG control +
Collagen 100µg/ml
Light Transmission
90s 90s

(ii) PECAM-1 XL +
Collagen 100 µg/ml
Collagen 100µg/ml
Light Transmission
90s

(57) Abstract: Activation of PECAM-1 (platelet endothelial cell adhesion molecule-1) with for example a small molecule or antibody derivative provides a new therapeutic route for modifying platelet activation and thrombus formation which has utility in the treatment or prevention of cardiovascular diseases, such as thrombosis, stroke and vascular occlusion and in the treatment or prevention of homeostasis disorders. This gives a better approach to the treatment of these conditions than existing drugs as it activates a general inhibitory mechanism rather than merely blocking one arm of the activation system. A screen for activators of PECAM-1 comprises PECAM-1, an ectodomain of PECAM-1, the cytoplasmic tail of PECAM-1, the ITIM of PECAM-1, an active site of PECAM-1, a recombinant extracellular domain of PECAM-1, or a part of derivative thereof and means for detecting activation or cross-linking or phosphorylation or tyrosine phosphorylation of PECAM-1, an ectodomain of PECAM-1, the cytoplasmic tail of PECAM-1, the IPIM of PECAM-1, an active site of PECAM-1, a recombinant extracellular domain of PECAM-1, or a part or derivative thereof.

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PECAM-1 MODULATION

Field of the Invention

The invention relates to the modulation of the activity of platelet endothelial cell adhesion molecule-1 (PECAM-1, CD31) for the treatment of or for reducing the occurrence of cardiovascular conditions such as thrombosis, vascular occlusion
5 stroke and for the treatment of or for reducing the occurrence of haemostasis disorders.

Review of the Art Known to the Applicant(s)

Thrombosis, an aggregation of blood factors primarily platelets and fibrin with entrapment of cellular elements frequently causing vascular obstruction at the
10 point of its formation, is a remarkably prevalent problem. It underlies most deaths from cardiovascular disease (whether there is another underlying cause or not). The 2001 Heart and Stroke Statistical Update (American Heart Association) reports the prevalence of cardiovascular disease to be 1 in 5 Americans with 12.4 million Americans currently suffering from coronary heart disease (myocardial

infarction and angina pectoris) and 4.7 million suffer from the effects of stroke. In 1998 cardiovascular disease (which also includes the effects of high blood pressure and congestive heart failure) killed 915,619 people in America, corresponding to 1 in every 2.5 deaths.

- 5 Platelets initiate blood clotting and control the formation of clots and/or thrombi, rapid and complete activation of platelets at sites of tissue damage is ensured through numerous positive feedback pathways, mainly through the actions of mediators such as thromboxane A₂ and ADP that are released from activated platelets. The existence of such a rapid and reactive system emphasises the need
10 for effective regulation of platelet function in order to prevent disorders such as thrombosis and haemorrhage. Platelet reactivity is a controlled balance between positive and negative regulatory factors and signalling mechanisms.

- The use of reagents that target and block activatory platelet cell surface receptors is known, and has provided a successful approach in the development of anti-
15 thrombotic drugs. However, due to the complex nature of the homeostasis system and the many ways it can be activated, none of these drugs has produced either a cure or a complete solution to all thrombosis related conditions. At best, in some trials, mortality has been reportedly reduced by up to 25%, however numerous side effects are associated with existing treatments. As a result almost all these
20 drugs are used in combination depending on the clinical circumstances, which serves to highlight the need for a safe effective treatment for thrombosis and related cardiovascular conditions.

- Much attention has recently been focussed on the identification of the receptors and signalling pathways that lead to platelet activation, particularly on exposure to
25 collagen, thrombin and ADP.

Despite this intensive research activity the use of an agent which activates natural inhibitory receptors so as to modify, or inhibit platelet activation and thereby reduce thrombus formation has not previously been either suggested or attempted.

5 The inventors have identified a negative regulation system that is mediated via a cell-surface ITIM-bearing adhesion receptor, PECAM-1. PECAM-1 signalling results in a negative feedback on platelet activation pathways and thereby sets the threshold stimulation level for platelet activation in the absence of injury thus preventing thrombosis..

10 They have ascertained that activation of PECAM-1 with for example a small molecule or antibody derivative provides a new therapeutic route for modifying platelet activation and thrombus formation which has utility in the treatment or prevention of cardiovascular diseases, such as thrombosis, stroke and vascular occlusion and in the treatment or prevention of haemostasis disorders.

15 The present invention provides a far better approach to the treatment of these conditions than existing drugs as it activates a general inhibitory mechanism rather than merely blocking one arm of the activation system.

In its broadest aspect the invention provides a method of activating PECAM-1 for modifying or reducing or inhibiting platelet activation, or platelet aggregation, or platelet secretion.

20 In a preferred aspect the invention provides a method for activating or cross-linking or phosphorylating PECAM-1 for the treatment of or for reducing the occurrence of cardiovascular diseases such as thrombosis, vascular occlusion or stroke, or for the treatment of or for reducing the occurrence of haemostasis disorders.

25 In a further aspect the invention provides an activator for use in this method.

In a further aspect the invention provides an activator for the treatment of or for reducing the occurrence of cardiovascular diseases such as thrombosis, vascular occlusion or stroke, or for the treatment of or for reducing the occurrence of haemostasis disorders.

- 5 In another aspect the invention provides an activator for use in the manufacture of a medicament for the treatment of or for reducing the occurrence of cardiovascular diseases such as thrombosis, vascular occlusion or stroke, or for the treatment of or for reducing the occurrence of haemostasis disorders.

- In another aspect the invention provides a screen for activators of PECAM-1
- 10 comprising PECAM-1, an ectodomain of PECAM-1, the cytoplasmic tail of PECAM-1, the ITIM of PECAM-1, an active site of PECAM-1, a recombinant extracellular domain of PECAM-1, or a part or derivative thereof and means for detecting activation or cross-linking or phosphorylation or tyrosine phosphorylation of PECAM-1, an ectodomain of PECAM-1, the cytoplasmic tail
- 15 of PECAM-1, the IPIM of PECAM-1, an active site of PECAM-1, a recombinant extracellular domain of PECAM-1, or a part or derivative thereof.

Description of the Preferred Embodiment

The invention will now be described by way of example with reference to the accompanying experimental data and drawings in which:

- 20 Figure 1. Cross-linking of PECAM-1 inhibits collagen-stimulated platelet aggregation. (A) PECAM-1 cross-linking on platelet surfaces results in its tyrosine phosphorylation and does not stimulate platelet aggregation. (i) PECAM-1 was immunoprecipitated from washed human platelets under resting conditions or following PECAM-1 cross-linking. Proteins were separated by SDS-PAGE and
- 25 immunoblotted to detect phosphotyrosine residues (upper panel). Immunoprecipitation was verified by reprobing for PECAM-1 (lower panel). (ii)

PECAM-1 was cross-linked on washed platelets and aggregation was monitored using optical aggregometry. (B) (i) Platelets were incubated with isotype-matched control IgG for 5 minutes before the addition of F(ab')₂ cross-linker for 90 seconds and then were stimulated with collagen (100 mM (mg/mL)). Aggregation was monitored using optical aggregometry. (ii) Cross-linking of PECAM-1 inhibits collagen-stimulated platelet aggregation. Platelets were stimulated with collagen (100 mM (mg/mL)) for 90 seconds with and without first cross-linking PECAM-1 and platelet aggregation monitored by optical aggregometry. Data are representative of 3 separate experiments. Tyr(P), tyrosine phosphorylation; PECAM-1 XL, PECAM-1 cross-linking.

Figure 2. PECAM-1 cross-linking inhibits GPVI- and thrombin receptor-stimulated platelet aggregation.

(A) Platelets were stimulated with Cvx at (i) 15 nM (ng/mL) and (ii) 62.5 nM (ng/mL) with and without first cross-linking PECAM-1, and aggregation responses were monitored by optical aggregation (arrow indicates the addition of Cvx). (B) Platelets were stimulated with thrombin at (i) 0.05 U/mL and (ii) 0.1U/mL with and without prior cross-linking of PECAM-1. Platelet aggregation was monitored by optical aggregometry (arrow indicates the addition of thrombin). Treatment of platelets with isotype-matched control IgG before stimulation with Cvx (15 nM (ng/mL)) and thrombin (0.05 U/mL) is shown in A(i) and B(i), respectively. Data are representative of 3 separate experiments. PECAM-1 XL, PECAM-1 cross-linking.

Figure 3. Platelet-dense granule secretion is inhibited by PECAM-1 signalling.

Platelets were loaded with [³H]5-HT before stimulation with Cvx (62.5 nM [ng/mL]) or thrombin (0.1 U/mL). Where required, PECAM-1 was cross-linked prior to the addition of an agonist. Secretion of [³H]5-HT into cell medium was measured using scintillation spectrometry. [³H]5-HT release is expressed as a percentage of total tissue content after subtraction of basal secretion values. Results represent mean +/- SE (n = 3). Student's t-test was used to compare

PECAM-1 cross-linked and non-cross-linked sample for statistical significance.
(* indicates p-values <0.05. PECAM-1 XL, PECAM-1 cross-linking)

Figure 4. PECAM-1 signaling inhibits platelet protein tyrosine phosphorylation.

Platelet lysates were prepared in Laemmli buffer from nonstimulated platelets
5 (stirred with buffer alone) and platelets stimulated for 90 seconds with Cvx (15
nM [ng/mL]) or thrombin (0.05 U/mL). Before stimulation, PECAM-1 was cross-
linked in some samples (A) or platelets were incubated with isotype-matched
control IgG and cross-linker F(ab')₂ (B). Proteins were separated by SDS-PAGE
under reducing conditions and immunoblotted to detect protein tyrosine
10 phosphorylation. PECAM-1 XL, PECAM-1 cross-linking

Figure 5. PECAM-1 cross-linking inhibits the mobilization of calcium from

intracellular stores. Fura-2 AM loaded human platelets were stimulated with
either Cvx or thrombin, and the mobilization of calcium was measured
fluorometrically (arrow indicates the addition of agonist). Panel A) Treatment of
15 platelets with control IgG and cross-linker F(ab')₂ has no effect on Cvx- and
thrombin-stimulated calcium mobilization.

Figure 6: Panels B i and ii) show representative calcium responses for stimulation
with Cvx at 62.5 nM (ng/mL) and 15 nM (ng/mL), respectively, with and without
first cross-linking PECAM-1. Traces are representative of 3 separate experiments.

20 Panel (iii) shows PECAM-1-induced percentage reduction in peak levels of
intracellular calcium stimulated by 62.5, 31.25, and 15 nM (ng/mL) Cvx. Data
presented represents mean +/- SE (n=3).

Figure 7: Panels C i, and ii) show representative calcium responses for stimulation
with thrombin at 0.1 U/mL and 0.05 U/mL, respectively, with and without first

25 cross-linking PECAM-1. Traces are representative of 3 separate experiments.
Panel (iii) PECAM-1-induced percentage reduction in peak levels of intracellular

calcium stimulated by 0.5, 0.1, and 0.05 U/mL thrombin. Data represent mean +/- SE (n = 3). PECAM-1 XL, PECAM-1 cross-linking.

Platelet endothelial cell adhesion molecule-1 PECAM-1 is a 130 kDa membrane-spanning glycoprotein whose expression is restricted to several haematopoietic cell types including platelets, monocytes, neutrophils, certain T-lymphocytes and also vascular endothelial cells. The functions of PECAM-1 are diverse and include angiogenesis, vasculogenesis, integrin regulation, transendothelial migration of leukocytes, and T- and B-cell antigen receptor function, although the role of this molecule in platelets is presently unclear. When PECAM-1 was cloned it was assigned to the family of cell adhesion molecules on the basis of structural similarities. PECAM-1 is involved in adhesion although much attention has been recently been directed to studying its ability to participate in signal transduction. The cytoplasmic tail of PECAM-1 contains a conserved motif called an immunoreceptor tyrosine based inhibitory motif (ITIM), which underlies its signaling properties and is shared by a growing family of inhibitory receptors. These include the immunoglobulin G receptor FcγRIIB, the killer inhibitory receptors (KIR) and signal regulatory proteins (SIRPs), although PECAM-1 is the only ITIM-bearing receptor that has been reported to be expressed in platelets. It has therefore been proposed that PECAM-1 should be assigned to the Ig-ITIM family of receptors.

The ligand binding properties of PECAM-1 are complex. It has the capacity for homophilic interactions, and also heterophilic interactions with a number of molecules that include integrin $\alpha_v\beta_3$ and CD38, PECAM-1 becomes phosphorylated on tyrosine residues in response to a variety of stimuli that include PECAM-1 cross-linking, activation of the high affinity receptor for immunoglobulin E (FcεRI), shear and oxidative stress. The inventors have recently found that platelet activation via the collagen receptor glycoprotein GPVI (GPVI) and thrombin receptors result in PECAM-1 tyrosine phosphorylation which is not dependent on platelet aggregation and secretion, although tyrosine

phosphorylation is enhanced by aggregation. The tyrosine residues that become phosphorylated in PECAM-1 have been mapped and fall within the ITIM. Phosphorylated ITIMs recruit signalling molecules such as the tyrosine phosphatases SHP-1 and SHP-2 that bind to the motif via Src-homology 2 domain interactions. Indeed, both SHP-1 and SHP-2 have been shown to associate with tyrosine phosphorylated PECAM-1, and PECAM-1 ITIM phosphopeptides activate these phosphatases *in vitro*. Generally, these protein tyrosine phosphatases exhibit inhibitory effects by counteracting tyrosine kinase-dependent pathways, although SHP-2 has been shown to positively regulate growth factor receptor signalling.

Immunoreceptor tyrosine-based activatory motif – (ITAM) bearing receptors have been shown to have a critical place in the regulation of platelet function. Indeed the collagen receptor GPVI-FcR γ -chain complex signals through an ITAM present on the cytoplasmic tail of the FcR γ -chain. Several studies in other cell systems have provided evidence of an antagonistic relationship between ITAM and ITIM containing receptors when expressed on the same cell. An example of this is the receptor for IgG Fc γ RIIB (ITIM) that negatively regulates cell activation stimulated by Fc γ RIIA (ITAM).

PECAM-1 was stimulated through cross-linking using antibodies directed to the extracellular domain of the receptor. This strategy was chosen as the most specific manner to activate PECAM-1, and activation was confirmed since cross-linking stimulated its tyrosine phosphorylation. PECAM-1 cross-linking stimulates tyrosine phosphorylation (Figure 1) and association of SHP-2 (not shown) but does not itself cause platelet activation. Tyrosine phosphorylation of PECAM-1 on cross-linking was shown not to be dependent on integrin $\alpha_{IIb}\beta_3$ engagement. Cross-linking PECAM-1 for 90s prior to stimulation with collagen caused inhibition of platelet aggregation. At lower concentrations of collagen, aggregation was inhibited completely, but even at very high concentrations of collagen (100 μ g/ml) PECAM-1 activation caused a substantial inhibition of

aggregation. Since collagen is also able to bind other receptors on the platelet, including the integrin $\alpha_2\beta_1$, the effect of PECAM-1 cross-linking on GPVI-mediated platelet aggregation using the specific agonist Cvx was examined. Similar results were obtained, with complete inhibition of aggregation at lower concentrations of agonist (15ng/ml) and partial effects at higher concentrations (62.5ng/ml), indicating that PECAM-1 is a potent inhibitor of GPVI-mediated (ITAM) platelet activation.

Experiments were conducted to determine whether the inhibitory effect of PECAM-1 is restricted to signalling via ITAM containing receptors. On the contrary, PECAM-1 cross-linking was also found to inhibit thrombin-stimulated platelet aggregation. Although no inhibitory effect of PECAM-1 was observed at moderate concentrations of thrombin (0.5 and 1U/ml, results now shown) dramatic levels of inhibition were observed at very high concentrations of collagen (100 μ g/ml). PECAM-1 mediated inhibition of thrombin-stimulated aggregation was observed only at lower thrombin concentrations (complete inhibition with 0.05U/ml and slight inhibition at 0.1U/ml). Showing that PECAM-1 activation inhibits thrombin-stimulated platelet aggregation less efficiently than collagen-stimulated aggregation.

The effect of PECAM-1 cross-linking on platelet aggregation stimulated by other G protein-coupled receptor agonists was also examined. Aggregation in response to low concentrations of the thromboxane mimetic U46619 were also reduced by PECAM-1 signalling. In addition, preliminary work suggests that ADP-induced platelet aggregation at low agonist concentrations may also be affected.

The inventors have demonstrated that activation of PECAM-1 signalling by antibody-mediated cross-linking results in inhibition of collagen-mediated activation. They have also shown that the effects of the GPVI-selective agonist convulxin, are inhibited by activation of PECAM-1 signalling. Thrombin-stimulated activation has also been shown to be inhibited by the activation of

PECAM-1 signalling, indicating that the inhibitory effects of PECAM-1 are not restricted to the inhibition of ITAM-containing receptor signalling pathways.

This is consistent with PECAM-1 performing a negative regulatory role in the control of platelet activation stimulated by both ITAM- and non-ITAM-containing receptor agonists.

Even though previous studies by for example Duncan G S, Andrew D P, Takimoto H, et al: Genetic evidence for functional redundancy of platelet/endothelial cell adhesion molecule-1 (PECAM-1): CD31- deficient mice reveal PECAM-1-dependent and PECAM-1-independent functions. J.Immun. 1999; 162:3022-3030, and Patil S, Newman D K, Newman P J: PECAM-1 serves as an inhibitory receptor that modulates platelet responses to collagen. Blood. 2001; 97:1727-1732, have reported that thrombin and ADP stimulated platelet aggregation in PECAM-1-deficient mouse platelets is normal.

The observation that ITAM-mediated signalling is inhibited to a higher degree than non-ITAM-mediated signalling is further supported by experiments in platelets where PECAM-1 was co-ligated with FcγRIIA (an ITAM-containing immunoglobulin G receptor). Co-ligation results in inhibition of FcγRIIA-mediated platelet aggregation and intracellular calcium mobilisation stimulated by receptor cross-linking.

The inhibitory effects of PECAM-1 on stimulation with agonists such as the collagen, Cvx, thrombin, and the thromboxane mimetic U46619, suggest that PECAM-1 inhibits primary signalling events and also secondary stimulation by factors released by activated platelets. It is possible, therefore, that inhibition of receptors that cross-talk with GPVI-mediated signalling may contribute to the inhibitory actions of PECAM-1 on collagen- and convulxin-stimulated activation. This may also explain why the inhibitory functions of PECAM-1 on GPVI-mediated activation are more effective than on thrombin-mediated activation.

Having established that PECAM-1 cross-linking inhibits platelet function, the effect of this on some aspects of signal transduction was examined. Platelet activation was found to be accompanied by a concomitant inhibition of platelet protein tyrosine phosphorylation and decreased levels of calcium mobilisation

5 from intracellular stores. Platelet activation by the collagen receptor GPVI is dependent on tyrosine kinases, and consequently is associated with the rapid tyrosine phosphorylation of a wide variety of platelet proteins. Stimulation with thrombin results in protein tyrosine phosphorylation, but to a lesser degree. Cross-linking PECAM-1, which in itself does not alter protein tyrosine phosphorylation

10 levels, inhibits substantially the level of tyrosine phosphorylation that is induced by subsequent stimulation with Cvx or thrombin. This is consistent with the reduction in aggregation and secretion observed. The identities of the phosphoproteins whose phosphorylation is reduced on PECAM-1 cross-linking is currently under investigation.

15 Underlying the PECAM-1 mediated inhibition of platelet activation, is a significant level of inhibition of calcium release from intracellular stores. As seen with aggregation assays, at lower concentrations of Cvx and thrombin (15ng/ml ad 0.05U/ml, respectively) PECAM-1 signalling inhibits release almost completely, where a partial effect is observed at higher agonist concentrations.

20 Calcium mobilisation is stimulated through the intracellular generation of inositol 1,4,5-trisphosphate (IP₃) from phosphatidylinositol 4,5-bisphosphate by phospholipase C. It is well established that in platelets, stimulation with collagen leads to phosphorylation and activation of PLC γ 2 isoform, and thrombin signalling regulates PLC β . PECAM-1 cross-linking results in a substantial and

25 significant reduction in total inositol phosphates stimulated by Cvx and thrombin. Which suggests that PECAM-1 exerts its effects on collagen- and thrombin-stimulated signalling either upstream of the PLC isoforms, or on these enzymes themselves.

Methods

Materials – Horm-Chemie collagen (collagen-fibres from equine tendons) was purchased from Nycomed (Munich, Germany). Convulxin was purified from the venom of the rattlesnake (*Crotalus durissus terrificus*) as described previously.

5 The thromboxane mimetic U46619 and ADP was purchased from Sigma (Poole, UK). Anti-phosphotyrosine monoclonal antibody (4G10) was from Upstate Biotechnology (TCS Biologicals, Buckinghamshire, UK). Anti-PECAM-1 antibodies: monoclonal antibody HC1/6 was from Serotec (Oxford, UK); polyclonal anti-PECAM-1 (C-20) and monoclonal antibody AB468 were from
10 Autogen Bioclear Ltd. (Wiltshire, UK); monoclonal antibody PECAM 1.3 was kindly provided by Professor Peter Newman (The Blood Centre of Woutheastern Wisconsin, Milwaukee, WI, USA). Control mouse IgG, was purchased from Sigma (Poole, UK). Monoclonal antibody IV.3 was purified from hybridoma cell culture medium and F(ab')₂ fragments generated by pepsin digestion using
15 reagents purchased from Pierce (Perbio Scientific, Chester, UK). Horseradish peroxidase-conjugated secondary antibodies and the enhanced chemiluminescence detection system were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). Fura-2 AM was from Molecular Probes (Cambridge Bioscience, Cambridge, UK).

20 **Preparation and stimulation of Platelets:** Human platelets from drug-free volunteers were prepared on the day of the experiment by differential centrifugation and suspended in modified Tyrodes-Hepes buffer (134mM NaCl, 0.34mM Na₂HPO₄, 2.9mM KCl, 12mM NaHCO₃, 20mM Hepes, 5mM glucose, 1mM MgCl₂, pH7.3) to a density of 2 x 10⁸ cells/ml. For protein precipitation
25 experiments, platelets were resuspended at 8 x 10⁸ cells/ml in buffer containing 1mM EGTA to prevent aggregation. Stimulation of platelets (450 µl) with collagen, convulxin (Cvx) and thrombin (delivered in 50µl) was performed at 37°C in an optical platelet aggregometer (Chrono-log Corporation) with

continuous stirring (1200rpm). PECAM-1 activation was stimulated by incubation with anti-PECAM-1 antibodies (AB468, 1 μ /ml or PECAM 1.3, 10 μ g/ml) for 5 minutes, followed by incubation for 90s with F(ab')₂ fragments of anti-IgG secondary antibodies (30 μ g/ml) to cross-link. mAb AB468 was generated against full length PECAM-1 ectodomain. Control experiments were performed using an irrelevant isotype-matched antibody in place of AB468 or PECAM-1.3 and used at the same concentration. In some experiments the low affinity receptor for IgG was blocked by incubation with a saturating concentration (1 μ g/ml) of F(ab')₂ fragments of mAbIV.3 for 5 minutes. Saturating concentrations of mAbIV.3 antibody were established by determining the concentration of F(ab')₂ fragment that completely inhibited subsequent whole IgG-mediated Fc γ RIIA cross-linking and platelet activation. Platelet aggregation was determined by optical aggregometry.

Immunoprecipitation studies: Platelet stimulation was terminated by the addition of an equal volume of ice cold lysis buffer (2% [v/v] Nonidet P40, 20mM Tris, 300 mM NaCl, 10mM EDTA, 1mM phenylmethylsulfonyl fluoride, 2mM Na₃VO₄, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 1 μ g/ml pepstatin A, pH7.3). Detergent-insoluble debris was removed and the lysates were pre-cleared by mixing with protein A-Sepharose for 1 hour at 4°C (20 μ l of a 50% [w/v] suspension of protein A-Sepharose of Tris-buffered saline-Tween [TBS-T: 20mM Tris, 137 mM NaCl, 0.1% [v/v] Tween 20, pH7.6). Protein A-Sepharose was removed from the lysates before the addition of anti-PECAM-1 antibody (HC1/6, 1 μ g). Following rotation at 4°C for 1 hour, 0.5 μ l secondary antiserum was added (rabbit anti-mouse IgG) and mixed for a further 30 min. 25 μ l protein A-Sepharose suspension was added to each sample and mixing continued for 1 hour before washing the Sepharose pellet in lysis buffer followed by a wash with TBS-T, and the addition of Laemmli sample-treatment buffer. Proteins were separated by SDS-PAGE under reducing conditions using 10% gels and transferred to polyvinylidene difluoride membranes by semi-dry western blotting.

Immunoblotting: Membranes were blocked by incubation in 10% (w/v) bovine serum albumin (BSA) dissolved in TBS-T. Primary and secondary antibodies were diluted in TBS-T containing 2% (w/v) BSA, and incubated with membranes for 1 hour at room temperature. Blots were washed for 2 hours in TBS-T following each incubation with antibodies for 1 hour at room temperature, and then developed using an enhanced chemiluminescence detection system. Primary antibodies were used at a concentration of 1 µg/ml (anti-phosphotyrosine, 4G10; anti-PECAM-1, C-20) and horseradish peroxidase-conjugated secondary antibodies were diluted 1:10000.

5-Hydroxytryptamine (5-HT) secretion assay: Platelets were loaded with [³H]5-HT by incubation with 0.5 µCi/ml of platelet-rich plasma for 1 h at 37°C. Platelets were prepared from the platelet-rich plasma as described above. Stimulation of platelets was terminated by addition of an equal volume of 6% glutaraldehyde and microcentrifugation, and the level of [³H]5-HT release into the supernatant was determined by scintillation spectrometry. [³H]5-HT release was expressed as a percentage of the total tissue content following subtraction of release under basal conditions.

Measurement of $[Ca^{2+}]_i$ by spectrofluorimetry: Washed human platelets (prepared as above) were incubated at 2×10^9 cells/ml in calcium free Tyrodes-hepes buffer with 3 µM Fura-2 AM for 45 min. Platelets were washed once and resuspended at 2×10^8 cells/ml in modified Tyrodes-hepes buffer. Stimulation of platelets (450 µl) in the presence of 2 mM EGTA with Cvx and thrombin (delivered in 50 µl) was performed with constant stirring at 37°C in a luminescence spectrophotometer (LS-50B, Perkin Elmer) with excitation wavelengths of 340 nm and 380 nm. Fluorescence emission was measured at a wavelength of 510 nm. Where required, PECAM-1 was cross-linked prior to stimulation with agonist as described above. The ratio of emission values (excitation at 340/380 nm) was calculated and converted to calcium concentration using FLWinLab software (Perkin Elmer) utilising the equation $[Ca^{2+}]_i = K_d \times (R -$

$R_{\min} / R_{\max} - R) \times \text{SFB}$ (where R is emission ratio value (340/380nm). R_{\max} the maximum 340/380 ratio, was determined by lysing platelets with 25 μ M digitonin in the presence of 1mM CaCl_2 . The R_{\min} 340/380 ratio was obtained by addition of 2mM EGTA. K_d is the dissociation constant of the Fura-2/ Ca^{2+} complex (224nm) and SFB is the fluorescence ratio at 340/380nm of R_{\min} and R_{\max} .

Statistical Analysis: Determination of statistical significance was performed using Student's paired T-test. The results are expressed as the mean \pm standard error of the mean (S.E.M.).

Experimental Data

10 **Cross-linking PECAM-1 inhibits collagen-stimulated platelet aggregation:**

An antagonistic relationship has been reported between ITIM- and ITAM-containing receptors when expressed in the same cell. Since the platelet collagen receptor GPV1 signals through an ITAM present on the FcR γ -chain with which it is associated, the inventors investigated the effect of PECAM-1 signalling on platelet activation with collagen. PECAM-1 was activated by incubation with antibodies specific for the ectodomain of PECAM-1 (AB468 [Figure 1] or PECAM-1.3 [not shown]) and cross-linked with a secondary antibody [F(ab')₂ fragment]. This resulted in increased tyrosine phosphorylation of the protein, and did not result in stimulation of platelet aggregation (Figure 1A). Tyrosine phosphorylation of PECAM-1 was maintained on cross-linking in the presence of EGTA (1mM), RGDS peptide (0.5mM) and the γ -chain peptide of fibrinogen (100 μ M) added separately or altogether (not shown). This, together with the fact that these experiments were performed on washed platelets indicates that the tyrosine phosphorylation of PECAM-1 on cross-linking is not dependent on integrin $\alpha_{IIb}\beta_3$ engagement. The effect of PECAM-1 cross-linking for 90s prior to stimulation with collagen was found to have a marked inhibitory effect on collagen-stimulated platelet aggregation. At lower concentrations of collagen (e.g. 10 μ g/ml), cross-linking of PECAM-1 before agonist addition completely

abolished aggregation (data not shown). Figure 1B(ii) shows the marked inhibitory effect of PECAM-1 cross-linking on a very high concentration of collagen (10µg/ml). The use of an isotype-matched IgG control and cross-linker F(ab')₂, had no effect of PECAM-1 tyrosine phosphorylation (not shown) and

5 collagen-stimulated platelet aggregation [Figure 1B(i)]. Results are representative of 3 separate experiments. Similar results were obtained using the alternative anti-PECAM-1 antibody PECAM 1.3. In some experiments the low affinity receptor for IgG FcγRIIA was blocked prior to PECAM-1 cross-linking and agonist stimulation using a saturating concentration of F(ab')₂ fragments of mAbIV.3. The

10 inhibitory effect of PECAM-1 cross-linking was unaltered under these conditions. This clearly indicates that the inhibitory effect of PECAM-1 using antibodies is not due to activation of FcγRIIA.

PECAM-1 cross-linking inhibits GPVI- and thrombin receptor-mediated platelet aggregation: Given the marked effect of PECAM-1 signalling on

15 collagen-mediated platelet aggregation the inventors investigated whether this effect was restricted to GPVI-mediated signalling only. GPVI was stimulated using the selective agonist Cvx, a protein purified from the venom of the rattlesnake *Crotalis durissus terrificus*. Aggregation stimulated with 15ng/ml Cvx was completely inhibited by prior activation of PECAM-1 [Figure 2A(i)], and a

20 partial inhibitory effect was observed at higher concentrations of Cvx [31.25 and 62.5ng/ml, not shown and in Figure 2A(ii), respectively]. Similar results were observed on stimulation of platelets with the G protein coupled receptor agonist thrombin. Complete inhibition of aggregation at 90s stimulation was observed at a thrombin concentration of 0.05U/ml [Figure 2B(i) and a partial effect at 0.1U/ml

25 Figure 2B(ii)]. The use of an isotype-matched IgG control and cross-linker F(ab')₂ had no effect on Cvx- or thrombin-stimulated platelet aggregation [Figure 2A(i) and 2B(i)]. No inhibitory effect of PECAM-1 activation was observed at higher concentrations of thrombin (e.g. 0.5 and 1U/ml, data not shown). Results are representative of 5 separate experiments. Similar results were obtained using the

alternative anti-PECAM-1 antibody PECAM-1.3, and when FcγRIIA was blocked prior to PECAM-1 cross-linking.

Platelet secretion is inhibited by PECAM-1 signalling: Platelet activation is accompanied by secretion from dense-granules. Dense granule secretion was assessed by measuring the release of [³H]5-HT from pre-loaded washed platelets. Figure 3 shows the results of experiments to determine the effect of PECAM-1 cross-linking on [³H]5-HT secretion. A significant reduction in secretion was observed in platelets where PECAM-1 was activated before stimulation with Cvx (81.9% +/- 2.9 to 39.8% +/- 4.1, p=0.02, n=3) or thrombin (70.9% +/- 4.6 to 37.0% +/- 8.0, p=0.01, n=3). The use of an isotype-matched IgG control and cross-linker F(ab')₂ had no effect on Cvx- or thrombin-stimulated dense granule secretion (not shown). Experiments performed in the presence of mAb IV.3 to block the Fc receptor FcγRIIA produced similar results.

PECAM-1 inhibits platelet protein tyrosine phosphorylation: The effect of PECAM-1 cross-linking on GPVI- and thrombin receptor-stimulated signalling was also investigated. Platelets were stimulated with Cvx (15ng/ml) or thrombin (0.05U/ml) with or without prior cross-linking of PECAM-1 for 90s. Whole cell protein tyrosine phosphorylation levels were determined by immunoblotting. Cross-linking PECAM-1 alone had no effect on basal platelet tyrosine phosphorylation levels (Figure 4). Stimulation with Cvx (15ng/ml) or thrombin (0.05U/ml) caused an increase in the level of tyrosine phosphorylation of a broad range of proteins. In those samples where PECAM-1 signalling was stimulated by cross-linking before incubation with Cvx or thrombin, total tyrosine phosphorylation was reduced (Figure 4). The use of an isotype-matched IgG control and cross-linker F(ab')₂ had no detectable effect on Cvx- or thrombin-stimulated total tyrosine phosphorylation levels.

PECAM-1 inhibits the mobilisation of calcium from intracellular stores:

Stimulation of the collagen receptor GPVI and thrombin receptors leads to rapid intracellular mobilisation of calcium, an effect that is essential for secretion and aggregation. Intracellular calcium levels were measured fluorometrically using the calcium sensitive dye Fura-2 AM. Experiments were performed in the presence of 2mM EGTA to prevent the entry of extracellular calcium. Stimulation of platelets with Cvx and thrombin resulted in a rapid increase in the levels of intracellular calcium that declined over a period of approximately 5 minutes. The incubation of platelets with control antibody ad cross-linker F(ab')₂ caused no change in Cvx- and thrombin-stimulated intracellular calcium mobilisation (Figure 5). The cross-linking of PECAM-1 for 90s before incubation with Cvx (62.5ng/ml) or thrombin (0.1U/ml) resulted in a markedly reduced level of calcium mobilisation [Figure 6B(i) and 7C(i)]. At the lower concentrations of agonists used (Cvx, 15ng/ml; thrombin, 0.05U/ml) calcium mobilisation was almost abolished [Figure 6B(ii) and 7C(ii)]. The effect of PECAM-1 cross-linking on the reduction of peak intracellular calcium levels for a range of agonist concentrations is shown in Figure 6B(iii) and 7C(iii). A reduction of at least 50% in calcium mobilisation was observed at all of the concentrations of Cvx and thrombin tested. Similar results were obtained using the alternative anti-PECAM-1 antibody PECAM-1.3. Furthermore, experiments performed in the absence of extracellular EGTA indicate that PECAM-1 cross-linking does not inhibit agonist-induced influx of calcium (data not shown).

CLAIMS:

1. The activation of PECAM-1 for modifying or reducing or inhibiting platelet activation, or platelet aggregation, or platelet secretion.
2. The activation claimed in Claim 1 wherein the activation comprises cross-linking PECAM-1.
- 5 3. The activation claimed in Claim 1 or Claim 2 wherein the activation comprises antibody mediated cross-linking.
4. The activation claimed in Claim 3 wherein the antibody is specific for the ectodomain of PECAM-1.
- 10 5. The activation claimed in any preceding Claim further comprising a secondary antibody.
6. The activation claimed in any one of Claims 1 to 5 wherein the activation comprises phosphorylation of PECAM-1.
7. The activation claimed in Claim 6 wherein the phosphorylation occurs at the cytoplasmic tail of PECAM-1.
- 15 8. The activation claimed in either Claim 6 or Claim 7 wherein the phosphorylation occurs within the ITIM of PECAM-1.
9. The activation claimed in any one of claims 6 to 8 wherein PECAM-1 is phosphorylated at tyrosine residues.

- 10 The activation or cross-linking or phosphorylation of PECAM-1 claimed
in any of Claims 1 to 9 for the treatment of or for reducing the occurrence
of cardiovascular diseases such as thrombosis, vascular occlusion or
stroke, or for the treatment of or for reducing the occurrence of
5 haemostasis disorders.
11. The activation claimed in any one of Claims 1 to 10 wherein the activation
or cross-linking or phosphorylation of PECAM-1 modifies or inhibits or
decreases any one selected from the group comprising; total tyrosine
phosphorylation, platelet protein phosphorylation, platelet secretion from
10 dense granules, mobilisation of calcium from intracellular stores,
production of inositol phosphates, and regulation of integrin-linked kinase.
12. The activation or cross-linking or phosphorylation of PECAM-1 as
claimed in any one of Claims 1 to 11 for inhibiting or modifying or
reducing platelet activation stimulated by ITAM or non-ITAM containing
15 receptors or receptor agonists.
13. The activation or cross-linking or phosphorylation of PECAM-1 as
claimed in Claim 12 for inhibiting or reducing or modifying the activation,
aggregation or secretion of platelets in response to any one selected from
the group comprising; collagen, collagen related peptide (CRP),
20 convulxin, thrombin, ADP, thromboxane mimetics, U46619,
immunoglobulin G FcγRIIA (FcγRIIA), immunoglobulin E FcεRI
(FcεRI), tyrosine kinase, GPVI- mediated signalling, and thrombin
receptor mediated signalling.
14. A PECAM-1 activator for use in accordance with any preceding claim.
- 25 15. An activator as claimed in claim 14 wherein the activator is selected from

the group comprising; a small molecule, an antibody, an antibody derivative, an agonist, an antagonist, a ligand, a DNA sequence, a complementary DNA sequence, an antisense DNA sequence, a probe, a protein sequence, a recombinant extracellular domain or domains of PECAM-1, a catalyst,

5 shear, oxidative stress, FcεRI, the high affinity receptor for FcεRI, an activated form of the high affinity receptor FcεRI, FcγRIIA, the low affinity receptor for FcγRIIA and an activated form of the low affinity receptor FcγRIIA .

10 16. The activator claimed in any Claims 14 or 15 for the treatment of or for reducing the occurrence of cardiovascular diseases such as thrombosis, vascular occlusion or stroke, or for the treatment of or for reducing the occurrence of haemostasis disorders.

15 17. The activator Claimed in any one of Claims 14 to 16 for use in the manufacture of a medicament for the treatment of or for reducing the occurrence of cardiovascular diseases such as thrombosis, vascular occlusion or stroke, or for the treatment of or for reducing the occurrence of haemostasis disorders.

20 18. A screen for activators of PECAM-1 comprising PECAM-1, an ectodomain of PECAM-1, the cytoplasmic tail of PECAM-1, the ITIM of PECAM-1, an active site of PECAM-1, a recombinant extracellular domain or domains of PECAM-1, or a part or derivative thereof and means for detecting activation or cross-linking or phosphorylation or tyrosine phosphorylation of PECAM-1, an ectodomain of PECAM-1, the cytoplasmic tail of PECAM-1, the IPIM of PECAM-1, an active site of
25 PECAM-1, a recombinant extracellular domain or domains of PECAM-1, or a part or derivative thereof.

Figure 1

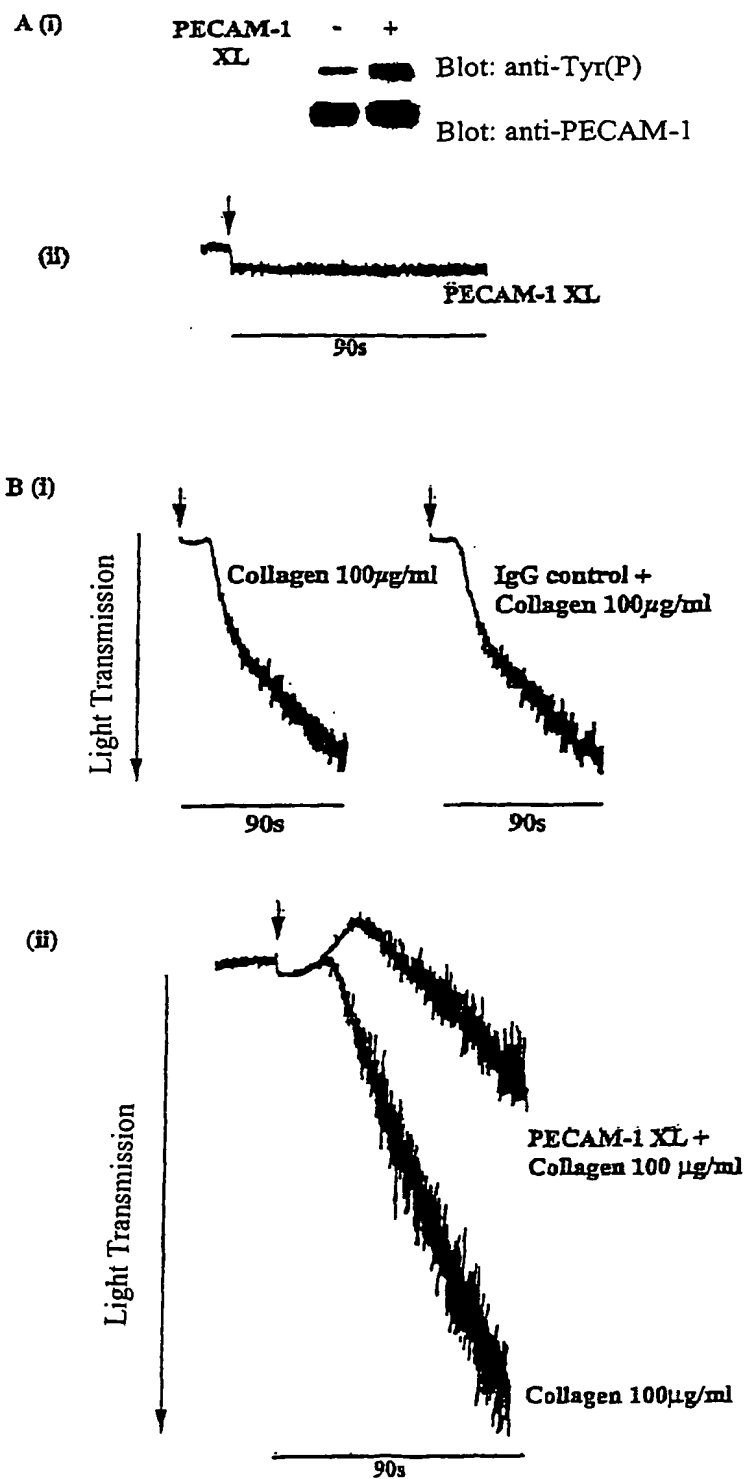


Figure 2

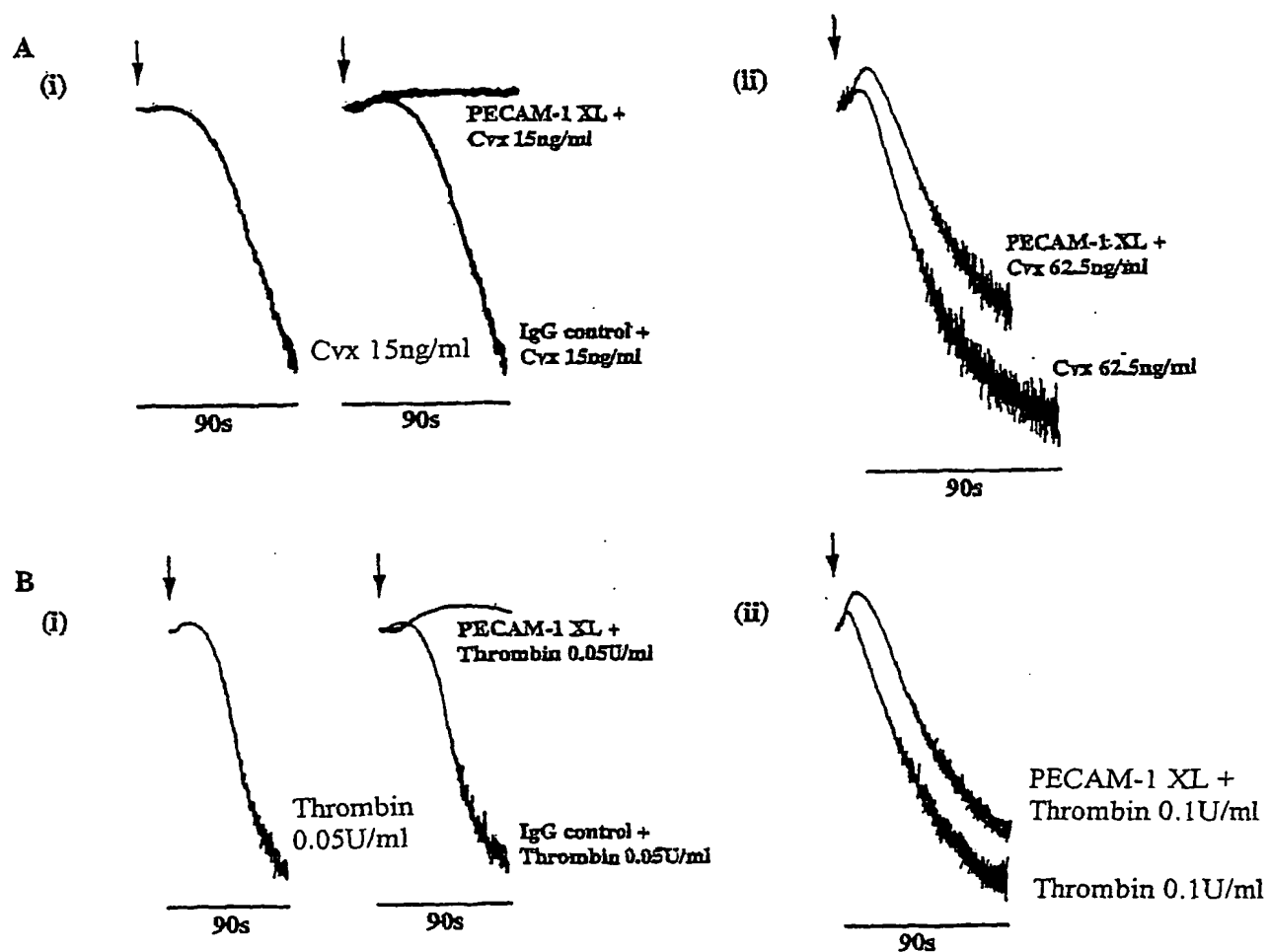


Figure 3

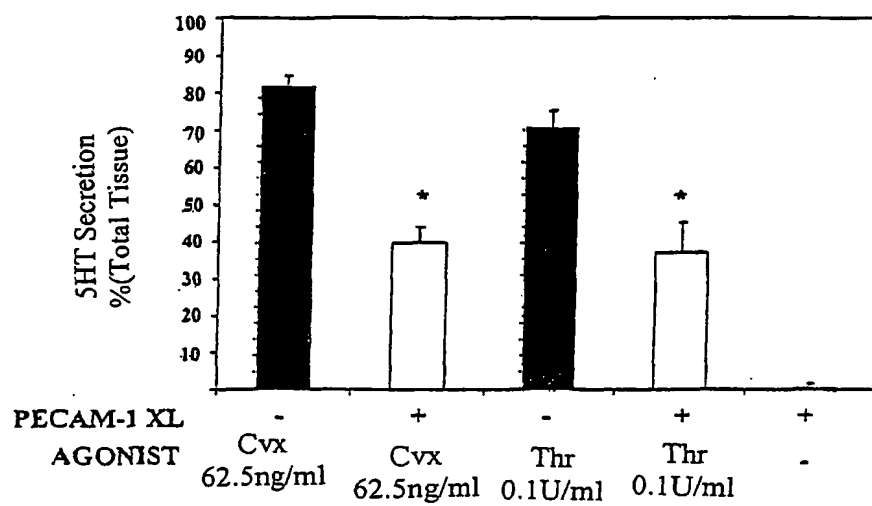


Figure 5

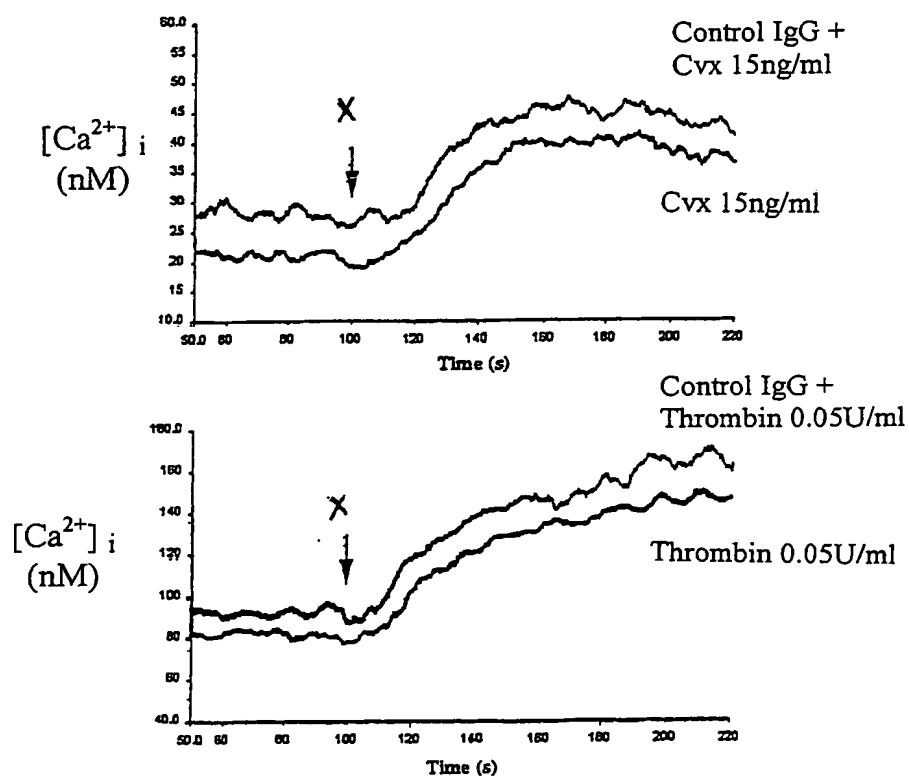
A

Figure 6

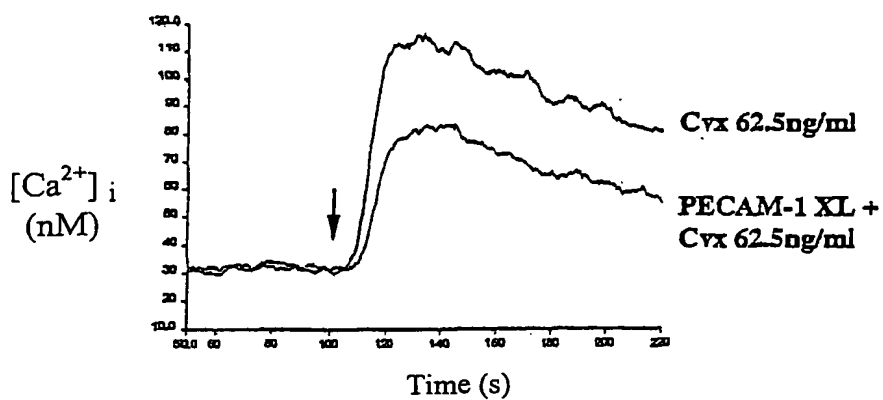
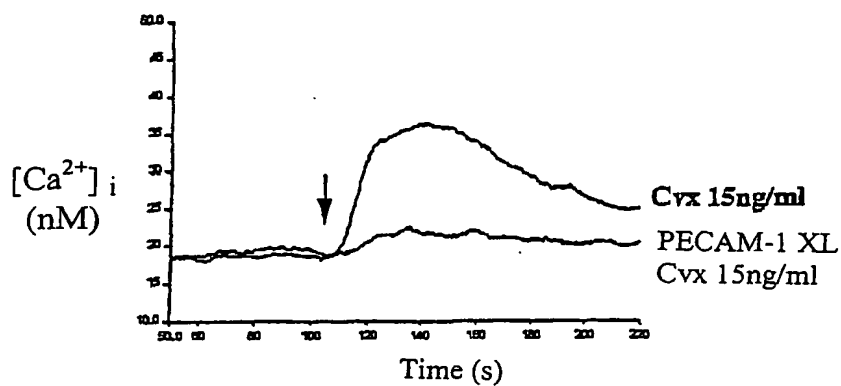
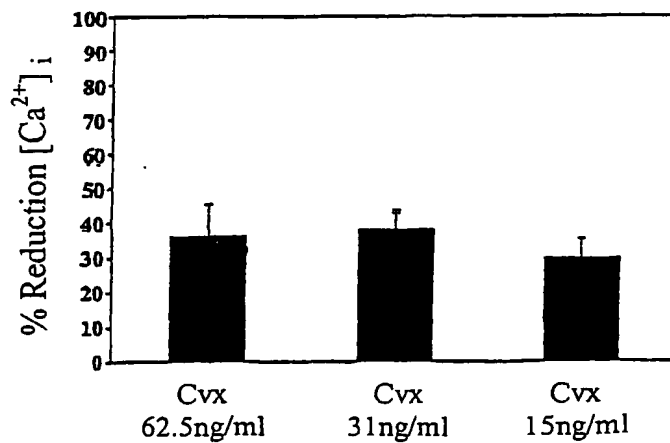
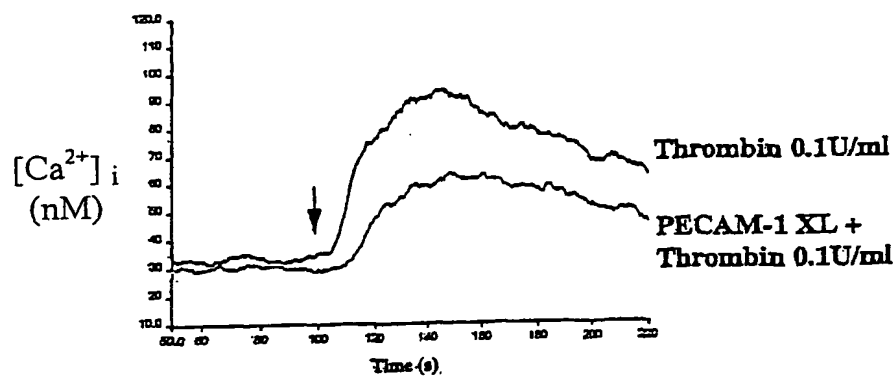
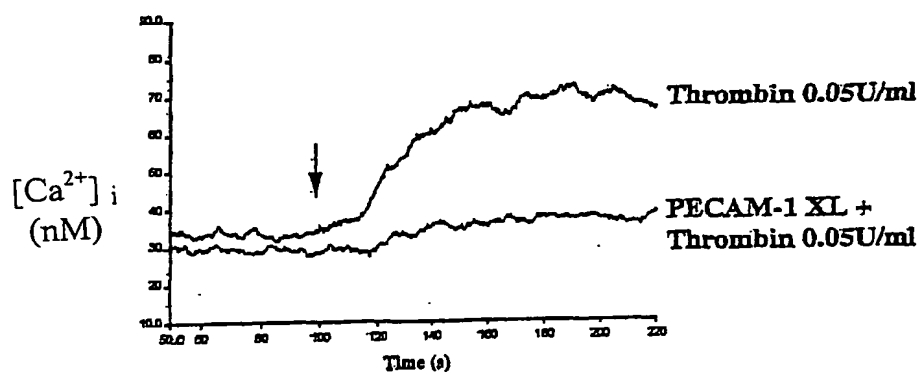
B (i)**(ii)****(iii)**

Figure 7

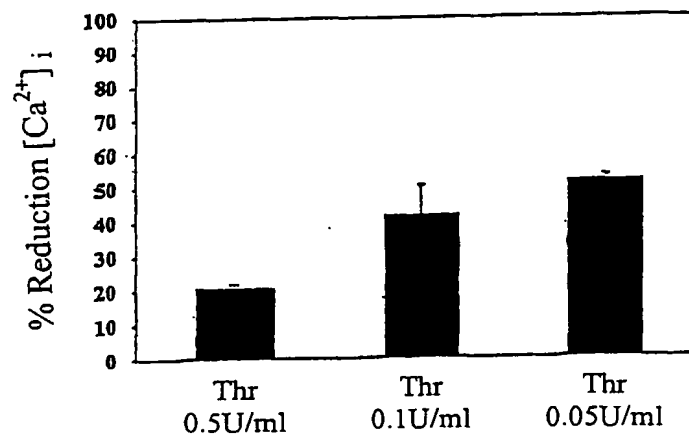
C (i)



(ii)



(iii)



INTERNATIONAL SEARCH REPORT

International Application No
PCT/65 J2/05730

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K39/395 C07K16/28 A61P7/00 A61P9/00 G01N33/50

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, EMBASE, WPI Data, PAJ, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	M. CICMIL ET AL.: "PECAM-1: a negative regulator of platelet activation?" BLOOD, vol. 96, no. 11 part 1, 16 November 2000 (2000-11-16), page 245A XP001148921 Philadelphia, PA, USA abstract# 1051	1,2,6-9, 11-13
Y	---	3-5,10, 16,17
	-/--	

☒ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
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- *P* document published prior to the international filing date but later than the priority date claimed

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X document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

G document member of the same patent family

Date of the actual completion of the international search

8 May 2003

Date of mailing of the international search report

21/05/2003

Name and mailing address of the ISA

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Nooij, F

INTERNATIONAL SEARCH REPORT

International Application No
PCT/Gb J2/05730

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	D. NEWMAN ET AL.: "Inhibition of antigen-receptor signaling by platelet endothelial cell adhesion molecule-1 (CD31) requires functional ITIMs, SHP-2, and p56lck." BLOOD, vol. 97, no. 8, 15 April 2001 (2001-04-15), pages 2351-2357, XP002240194 Philadelphia, PA, USA figures 2,4,6	14,15,18
Y	-----	3-5
X	D. NEWTON-NASH ET AL.: "A new role for platelet-endothelial cell adhesion molecule-1 (CD31): Inhibition of TCR-mediated signal transduction." THE JOURNAL OF IMMUNOLOGY, vol. 163, no. 2, 15 July 1999 (1999-07-15), pages 682-688, XP002240195 Baltimore, MD, USA abstract page 683, right-hand column, line 6 - line 42 figure 2	14,15,18
X	----- P. EVANS ET AL.: "Signaling through CD31 protects endothelial cells from apoptosis." TRANSPLANTATION, vol. 71, no. 3, 15 February 2001 (2001-02-15), pages 457-460, XP008016872 Baltimore, MD, USA abstract page 459, right-hand column, line 9 -page 460, left-hand column, line 9	14-16,18
Y	----- R. GUMINA ET AL.: "Antibody to platelet/endothelial cell adhesion molecule-1 reduces myocardial infarct size in a rat model of ischemia-reperfusion injury." CIRCULATION, vol. 94, no. 12, 15 December 1996 (1996-12-15), pages 3327-3333, XP008016839 New York, NY, USA abstract	10,16,17

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 02/05730

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 1-9 and 11-13 (all partially, as far as an in vivo method is concerned) and claim 10 (completely) are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☒ Claims Nos.: 14-17 (partially)
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 14-17 (partially)

Present claims 14-17 relate to an extremely large number of possible compounds. In fact, the claims contain so many options that a lack of clarity (and/or conciseness) within the meaning of Article 6 PCT arises to such an extent as to render a meaningful search of the claims impossible.

Furthermore, said present claims relate to compounds defined by reference to a desirable characteristic or property, namely an agonist, antagonist or catalytic activity. The claims cover all compounds having these characteristics or properties, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such compounds. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the compound by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible.

Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely anti-PECAM-1 antibodies as described in the sections 'methods' and 'experimental data' of the description of the present underlying application.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

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